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Novel Allele of *FAD2-1A* from an EMS-Induced Mutant Soybean Line (PE529) Produces Elevated Levels of Oleic Acid in Soybean Oil

Hyun Jo [1,†], Changwan Woo [1,†], Nabachwa Norah [2], Jong Tae Song [1] and Jeong-Dong Lee [1,3,*]

[1] Department of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 41566, Korea
[2] Department of Food Security and Agriculture Development, Kyungpook National University, Daegu 41566, Korea
[3] Department of Integrative Biology, Kyungpook National University, Daegu 41566, Korea
* Correspondence: jdlee@knu.ac.kr; Tel.: +82-53-950-5709; Fax: +82-53-958-6880
† These authors contributed equally to this work.

Abstract: Soybean seed oils contain approximately 23% oleic acid, and elevated amounts of oleic acid help prevent cardiovascular diseases and improve the quality of the oil. Chemically, it helps maintain the oxidative stability of oil; hence, soybean breeders primarily seek to increase its concentration for improved oil quality. As soybean seeds with mutant alleles of *FAD2-1A* and *FAD2-1B* genes have been reported to produce approximately 80% of oleic acid, a mutant population was developed from an ethyl methanesulfonate (EMS)-induced soybean cultivar (Pungsannamul). From this, a new mutant allele of *FAD2-1A* was identified using mutant lines with elevated oleic acid levels and the pooled-DNA sequencing method. This study identified PE529 as the allele with >40% oleic acid carrying the novel allele of the *FAD2-1A* gene. The single nucleotide polymorphism (SNP) in PE529 also induced the conversion from tryptophan to a premature stop codon at position 293 in the amino acid sequence (W293STOP). The inheritance analysis showed that the elevated oleic acids in PE529 were attributed to the *fad2-1a* W293STOP allele. In this study, seeds capable of producing approximately 80.0% oleic acid were identified from F_2 populations where *fad2-1a* W293STOP and *fad2-1b* alleles were segregated. Hence, soybeans with novel alleles are useful genetic resources to improve soybean oil quality in breeding programs.

Keywords: *FAD2-1*; high oleic acid oil; EMS; soybean; genetic resource

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1. Introduction

Soybean (*Glycine max* (L.) Merr.) is an economically important legume crop for vegetable oil, protein meal, and soybean food. In 2021, 28.7% (58.7 million metric tons) of global vegetable oil consumption was sourced from soybeans [1]. The fatty acid profiles in soybean oil are primarily used in food applications such as cooking, baking, frying, and some industrial applications such as biodiesel production [2]. Soybean oil contains five main fatty acids: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3). All of which are hydrocarbon chains esterified to glycerol to form triacylglycerol, however, among these, 16:0 and 18:0 fatty acids are saturated fatty acids that do not contain double bonds in their hydrocarbon chains, whereas oleic acid (18:1), 18:2, and 18:3 fatty acids are unsaturated fatty acids that contain double bonds. Of the unsaturated fatty acids, 18:2 and 18:3 are polyunsaturated, whereas oleic acid (18:1) is a monounsaturated fatty acid. Commodity soybean oil contains 11% 16:0, 4% 18:0, 23% 18:1, 54% 18:2, and 8% 18:3 fatty acids, respectively [3].

The polyunsaturated fatty acid content in soybean oil determines the oil quality. Although polyunsaturated fatty acids are essential to the human diet, it inversely affects the

oxidative stability in oil, in which higher contents reduce the oxidative stability. This results in undesirable flavors, odors, and the production of trans-fat during the hydrogenation process [4]. Specifically, trans-fat harms human health, as it increases the risk for coronary heart diseases and complications arising from high cholesterol, based on previous clinical studies [5–8]. Considering this, research efforts, including those in breeding programs, have focused on decreasing the polyunsaturated fatty acid content to approximately 80% monounsaturated fatty acid (oleic acid) to address health issues while maintaining the oxidative stability in soybean oil and improving oil quality [9–14].

The microsomal delta-12 fatty acid desaturase 2 (FAD2) is an enzyme involved in the conversion of oleic to linoleic acid in the lipid biosynthesis pathway [4,7]. Specific mutations in the *FAD2* genes contribute to increasing the oleic acid content in different crops, including safflower [15], sunflower [16], peanut [17–19], canola [20,21], cotton [16], and maize [5], as identified in previous studies. Here, a single mutant allele of the *FAD2* gene showed elevated oleic acid concentrations in seed oil. Particularly, in cotton and soybean, the silencing of all *FAD2* genes in seed development stages reduced the enzyme activity of FAD2, which resulted in approximately 80% of oleic acid being produced in these crops [16,22,23]. In peanut oil, the combination of mutations in *ahFAD2A* and *ahFAD2B* genes also produced higher oleic acid concentrations [18,19].

Owing to soybean genome duplication, two identical copies of *FAD2* genes, *FAD2-1* and *FAD2-2*, were successfully identified [24,25]. The soybean genome is composed of two *FAD2-1* and five *FAD2-2* members, namely: *FAD2-1A* (*Glyma.10G278000*), *FAD2-1B* (*Glyma.20G111000*), *FAD2-2A* (*Glyam.19G147300*), *FAD2-2B* (*Glyma.19G147400*), *FAD2-2C* (*Glyma.03G144500*), *FAD2-2D* (*Glyma.09G111900*), and *FAD2-2E* (*Glyma.15G195200*) [7,26]. Based on previous studies, the *FAD2-1A* and *FAD2-1B*, which are mainly expressed in the developing seed stages, were primarily responsible for the elevated oleic acid concentrations in soybean seed [7,8]. Notably, these genes are closely related as both consist of two exons and an intron, with a 99% identity in the amino acid sequences [7,8].

Using the X-ray mutagenesis of the Bay soybean cultivar (27.2% oleic acid), two mutant soybean genotypes, M23 (48.4% oleic acid) and KK21 (47.2% oleic acid), particularly the different mutant alleles in the *FAD2-1A* gene, were found to cause the increase in the oleic acid concentration, [13,27,28]. The development of soybean mutants M23 and KK21 can be attributed to the deletion of 160 kb and a single nucleotide in the *FAD2-1A* gene, respectively. The result of 17D from the Williams 82 ethyl methanesulfonate (EMS)-induced population has been reported to contain a missense mutation in the *FAD2-1A* gene (S117N), which caused the elevated oleic acid concentration in soybeans [29]. Recurrent selection also resulted in the development of mid-oleic acid soybean lines such as CR03-529 (38.3% oleic acid), N98-4445A (54.9% oleic acid), and N97-3393-3 (49.8% oleic acid) [30,31]. Meanwhile, soybean germplasms of PI 567189A and PI 283327 were similarly found to elevate oleic acid concentrations due to missense mutations in the *FAD2-1B* gene as well (S86F, M126V, P137R, and I143T) [26]. As soybean breeding has been known to combine natural and induced mutations in *FAD2-1A* and *FAD2-1B* genes, approximately 80% oleic acid can be produced using conventional methods [26,29,32]. Meanwhile, in commercial high-oleic acid soybean cultivars such as Plenish®, Vistive Gold®, and Calyxt high-oleic soybeans, transgenic and genome editing approaches have been developed to downregulate the expression level of *FAD2-1* genes [22,23,33].

The application of chemical mutagenesis has been successful in expanding genetic diversity in numerous crops, including soybean [13,29]. In this study, a mutant population was developed from the EMS-induced Korean soybean cultivar (Pungsannamul) [34,35] to identify a novel mutant allele of *FAD2-1A* and determine the feasibility of producing high oleic acid concentrations by combining it with the reported *fad2-1b* P137R from PI 283327 in previous studies [26]. Mutant lines with elevated oleic acid levels were selected using a reverse genetic approach as a pooled-DNA sequencing method.

2. Materials and Methods

2.1. EMS-Induced Mutation Population

In our previous study, a mutant population consisting of the M_4 generation was developed by EMS mutagenesis of the cultivar Pungsannamul [34,35]. Fatty acid profiles of 2281 mutant lines were determined, and of these mutant lines, 48 lines with elevated oleic acid concentrations were selected to identify novel alleles of the *FAD2-1A* and *FAD2-1B* genes (Table S1). Williams 82 and Pungsannamul were used as control cultivars for fatty acid analyses.

2.2. Fatty Acid Composition Determination

Mutant lines derived from the EMS treatment of the cultivar Pungsannamul were subjected to fatty acid phenotyping using gas chromatography (GC). Fatty acid profiles of each of the soybean lines expressed as the percentage of each fatty acid were obtained using an Agilent series 7890A capillary GC equipped with an ionization detector (Agilent Technologies Inc., Wilmington, DE, USA). Oil was extracted from crushed seeds and stored in l mL of extraction solution (chloroform: hexane: methanol [8:5:2 v/v]) for ~12 h. Approximately 100 µL of the extracted solution was added to 75 µL of methylation reagent comprised of 0.25 M methanolic sodium methoxide: petroleum ether: ethyl ether [1:5:2, $v/v/v$]. Thereafter, the derivative was diluted with hexane to obtain a total volume of 1 mL. The five fatty acids obtained were then separated on a DB-FFAP capillary Agilent column (30 m × 0.25 mm, 0.25 µm, Agilent Technologies Inc., Wilmington, DE, USA). Standard fatty acid mixtures (Fame #16, Restek) were used for reference calibration.

2.3. FAD2-1A and FAD2-1B Sequencing

Forty-eight mutant lines with elevated oleic acid concentrations were used for Sanger sequencing to identify variations in the *FAD2-1A* and *FAD2-1B* genes. Using the HiGene Genomic DNA Prep Kit (BioFACT Co., Daejeon, Korea), genomic DNA was isolated from each mutant line with phenotypically elevated concentrations of oleic acid, including the wild type of Pungsannamul. The quality of genomic DNAs was checked in 1% agarose gel. Individuals were used as templates for polymerase chain reaction (PCR) amplification of the coding regions of *FAD2-1A* and *FAD2-1B* genes. PCR was performed in a 20 µL reaction with a composition of 2 µL template DNA, 2 µL 10× Diastar buffer, 0.1 µL Diastar taq DNA polymerase (SolGent Co., Daejeon, Korea), 0.4 µL dNTP, and 2 µL of forward and reverse primers. Four sets of primers for *FAD2-1A* and *FAD2-1B* genes were used (Table S2). PCR amplicons for the *FAD2-1A* gene were conducted under the following conditions: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 50 s. PCR amplicons for the *FAD2-1B* gene were conducted under the following conditions: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 50 s. The size of PCR amplicons was verified using 1% agarose gel electrophoresis. The PCR amplicons of the 48 mutant lines were then purified using a Solg Gel & PCR purification kit (SolGent Co., Daejeon, Korea) and pooled using a combination of 8–10 PCR amplicons to make into 5 groups (Table S1). Each pooled PCR amplicon was sequenced using ABI 3730XL DNA Analyzer (50 cm capillary) in the SolGent company. Sequence alignments were performed using multiple sequence alignments from the CLUSTALW program (http://www.genome.jp/tools-bin/clustalw accessed on 1 August 2022). Finally, to identify nucleotide variations, sequences were aligned with those of Williams 82 and Pungsannamul.

2.4. The Development of FAD2-1A W293STOP Genotyping Assay

Simpleprobe, obtained from Fluoresentric, Inc. (Park City, UT, USA), was designed using the Lightcycler Probe Design Software 2.0 (Version 1, Roche Applied Sciences, Penzberg, Germany) by inputting the target region of the sequence with a single highlighted mutant position. The *FAD2-1A* W293STOP genotyping assay was conducted with an asymmetric mixture of primers [0.5 µM forward (5′-CCGTGTTGCAACCCTGAAAG-3′) and 0.2 µM reverse (5′-ACCATGATCGCAACAAGCTG-3′) with 5:2 asymmetric mix of primers]; the

FAD2-1A W293STOP SimpleProbe was used at a concentration of 2 µM of 5′-FAM-SPC-CCAAAGCTCCCTTCAGCCAG-phosphate-3′. The total volume of reactions used was 20 µL, which contained 5–50 ng DNA template, primers, buffer (40 mM Tricine-KOH (pH 8.0), 16 mM KCl, 3.5 mM $MgCl_2$, 3.75 ug mL^{-1} BSA, 200 µM dNTPs), 5% DMSO, SimpleProbe, and 0.2 X Titanium Taq polymerase (BD Biosciences, Palo Alto, CA). Assay reactions were conducted in a 96-well plate in a Roche LightCycler 480 II using the following reaction conditions: 95 °C for 3 min, followed by 50 cycles of 95 for 20 s, 60 °C for 20 s, 72 °C for 20 s, and a melting curve from 50–72 °C. The fluorescence was read every 0.1 °C within 50–72 °C, and the single nucleotide match or mismatch was determined using an integration of the fluorescence disappearance and increasing melting temperature. The homozygous wild-type allele of *FAD2-1A* was detected at 64 °C, indicated by a peak: the homozygous *fad2-1a* W293STOP showed a peak at 57 °C, while heterozygous alleles produced both peaks.

2.5. Development, Phenotyping, and Genotyping of Segregating Populations

In order to investigate the inheritance of a mutant allele from PE529 and produce approximately 80% oleic acid when combined with *fad2-1b* P137R allele from PI 283327, four different cross populations were developed using PI 283327 and three Korean soybean cultivars as Sunpung, Pungsannamul, and Hosim. Sunpung and Pungsannamul were observed to have normal oleic acid concentrations, whereas Hosim produced approximately 80% oleic acid, owing to the combination of *fad2-1a* S117N from 17D and *fad2-1b* P137R from PI 283327 [26,36]. The four cross populations were developed from the crosses of the accessions: population 1 from Pungsannamul and PE529, population 2 from Sunpung and PE529, population 3 from PE529 and PI 283327, and population 4 was developed from Hosim and PE529. Initial crosses were performed during the summer of 2021 at the affiliated field located within Kyungpook National University (Gunwi, 36°14′ N, Republic of Korea). In order to produce F_2 seeds, F_1 plants of the crossing populations were grown in a greenhouse during 2021–2022. Half of the produced F_2 seeds were then used to determine fatty acid profiles, while the remaining were ground in liquid nitrogen for genomic DNA isolation. PCR amplification and genotyping assays for the 17D *fad2-1a* (S117N) and PI 283327 *fad2-1b* (P137R) alleles were performed as described by Pham et al. [26].

2.6. Statistical Analysis

The IBM SPSS program (SPSS for Windows, Standard version 26.0, SPSS, Chicago, IL, USA) was used for all analyses of oleic acid content in the soybean seeds. The frequency distribution of fatty acid concentration in the 2281 EMS population was determined using descriptive statistics. Analysis of variance (ANOVA) was then conducted to determine differences among genotypic groups for oleic acid concentrations based on a combination of mutant alleles of the *FAD2-1A* and *FAD2-1B* genes. The least significant difference (LSD) was used for the multiple comparison analysis of genotypic groups, and results were reported at a significance level of 5%. Chi-square analysis was carried outperformed to identify the goodness-of-fit of the observed to expected ratios of genotypes from each population.

3. Results

3.1. Phenotypic Distribution of a Mutation Population

This study evaluated seed fatty acid concentrations in a mutant population consisting of 2281 lines (Figure S1). The wild-type, Pungsannamul showed fatty acid concentrations of 11.6%, 2.8%, 23.0%, 55.1 and 7.5% of the 16:0, 18:0, oleic acid (18:1), 18:2, and 18:3 fatty acids, respectively. However, in the same order, the fatty acid variation in the mutant lines ranged from 8.2–16.1%, 1.8–11.3%, 11.5–47.5%, 30.2–61.2%, and 5.9–17.3%. Of a total of 2281 mutant lines, 48 mutant lines were identified to have elevated oleic acid concentrations (Table S1). The oleic acid in the 48 mutant lines ranged from 29.1–49.1% in soybean seeds, in which PE529 had the highest concentration (49.1%), or 2-folds greater than that of Pungsannamul.

3.2. Pooled-DNA Sequencing of FAD2-1A and FAD2-1B Genes

Forty-eight mutant soybean lines were observed to produce elevated concentrations of oleic acid in the soybean seeds. Here, a new allele of the *FAD2-1A* gene was found based on the pooled PCR amplicons that sequenced a combination of 8–10 individuals (Table S1). In one of the pooled PCR amplicons, double peaks of chromas DNA sequences, consisting of eight individuals: PE529, PE908, PE1326, PE1759, PE1889, PE1470, PE1962, and PE2166, were identified in the sequences of the *FAD2-1A* gene (Figure 1a). However, a variant of the *FAD2-1B* gene was not identified across pooled DNA samples. In the sequencing of each mutant line with a part of the *FAD2-1A* gene to align with the sequences of Williams 82 and Pungsannamul (Figure 1b), the mutant line of PE529 was found to have a mutation in the *FAD2-1A* gene in which it contained elevated oleic acid concentrations. Further, PE529 was identified to carry an SNP (G50014969A in Wm82.a2.v1) in the coding sequence, resulting in a change from a tryptophan codon (TGG) to a premature stop codon (TGA) at amino acid 293 (Figure 1c).

3.3. Inheritance of FAD2-1A W293STOP Allele in PE529

The result of the inheritance analysis showed that two segregating populations developed from the crosses of populations one and two. Hence, 100 F_2 seeds from each segregating population were analyzed for their oleic acid concentrations, and the genomic DNA of F_2 individuals from these populations was isolated to determine the genotype with a molecular marker and the corresponding association of its phenotype and genotype (Figure S2). Results showed the genotype of F_2 individuals had a segregation ratio of 1:2:1 based on the chi-square analysis (Table 1). For population one, the oleic acid content of F_2 progeny lines with homozygous *FAD2-1A*/*FAD2-1A*, heterozygous *FAD2-1A*/*fad2-1a*, and homozygous *fad2-1a*/*fad2-1a* ranged from 16.7–35.1%, 19.7–42.7%, and 31.2–53.4%, respectively (Figure 2a). The mean oleic acid of F_2 individuals with homozygous *fad2-1a* W293STOP allele in population one was 40.3%, which was significantly higher than that of other genotypic groups (Figure 2a). Meanwhile, the oleic acid content in population two ranged from 19.3–38.5%, 21.8–43.0%, and 34.3–58.3% for homozygous *FAD2-1A*/*fad2-1a*, heterozygous *FAD2-1A*/*fad2-1a*, and homozygous *fad2-1a*/*fad2-1a*, respectively (Figure 2b). Here, the mean oleic acid of F_2 individuals with the homozygous *fad2-1a* W293STOP allele in population two was significantly higher than that of other genotypic groups (Figure 2b). Therefore, the *fad2-1a* W293STOP allele is inferred to be a new recessive allele responsible for the elevated oleic acid concentrations in PE529.

Table 1. Genotypic ratio of two F_2 populations.

Population	Progeny	Observed for Genotypic Ratio			Expected for Genotypic Ratio			Chi-Square (1:2:1)	*p*
		FAD2-1A/FAD2-1A	***FAD2-1A/fad2-1a***	***fad2-1a/fad2-1a***	***FAD2-1A/FAD2-1A***	***FAD2-1A/fad2-1a***	***fad2-1a/fad2-1a***		
Pungsannamul × PE529	F_2 (n = 100)	26	45	29	25	50	25	1.18	0.554
Sunpung × PE529	F_2 (n = 100)	29	47	24	25	50	25	0.86	0.651

FAD2-1A indicates functional microsomal delta-12 fatty acid desaturase 2 gene which is similar to the Williams 82 reference sequence for *Glyma.10G278000*. The *fad2-1a* indicates mutant allele of *FAD2-1A* W293STOP from PE529. Pungsannamul and Sunpung are Korean soybean cultivars with normal oleic acid concentrations and contain functional *FAD2-1A* gene.

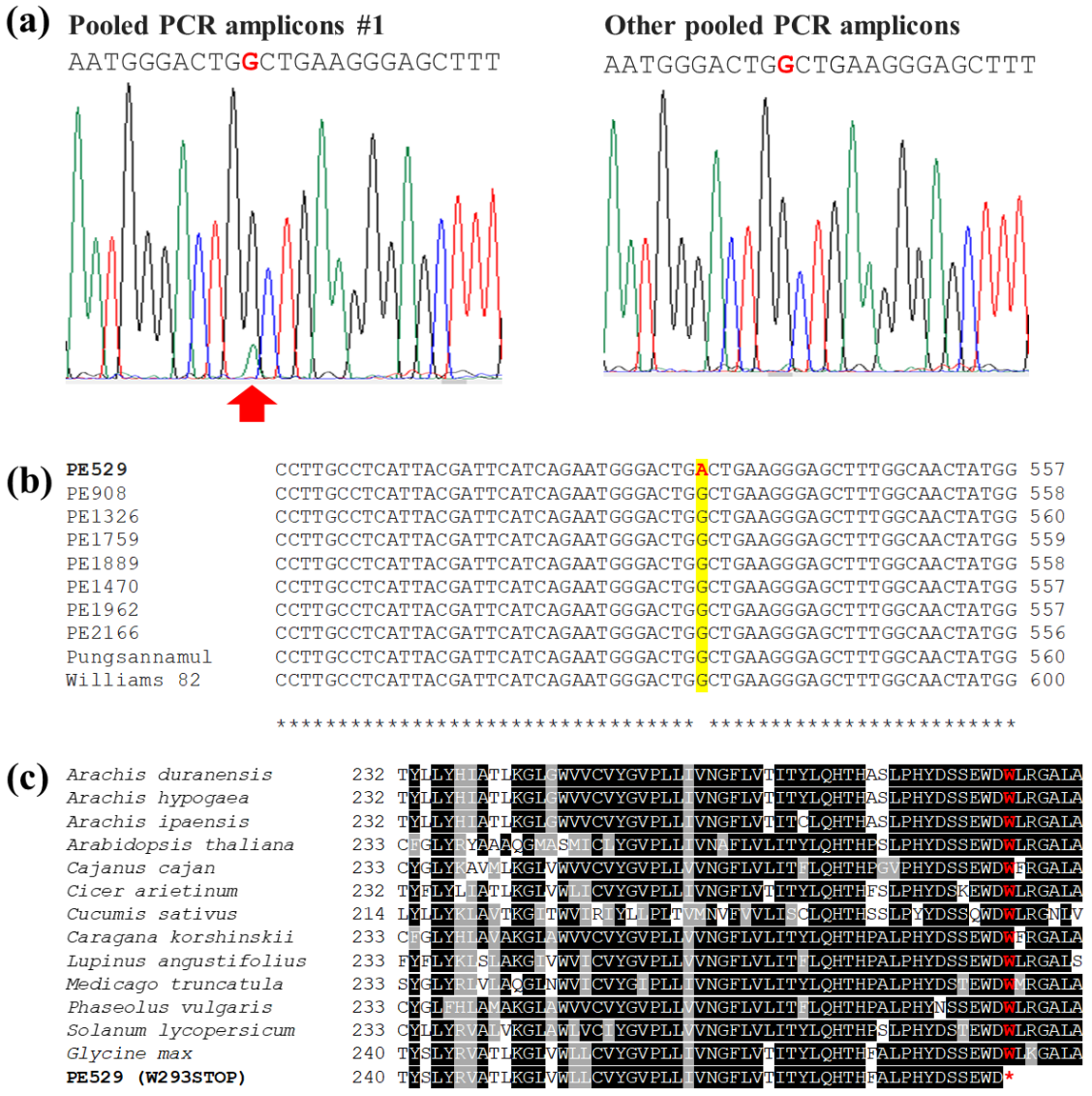

Figure 1. Characterization of mutant allele of *FAD2-1A* gene in a soybean mutant line PE529. (**a**) Chromas DNA sequence traces for the pooled PCR amplicons. The peaks correspond to the DNA sequence information from the Sanger sequence analysis. The positions of the DNA sequence change are indicated above the trace by the red highlight. A red arrow indicates a possible novel allele of *FAD2-1A* gene from one of pooled PCR amplicons (**b**) Multiple alignment of *FAD2-1A* genes. ClustalW sequence alignment of part of *FAD2-1A* gene with 8 mutant lines, Pungsannamul, and Williams 82 which confirm the new SNP position. (**c**) Multiple alignment of *FAD2-1A* genes in different plant species. Identical amino acid position is highlighted in black, and position of amino acid change is indicated as red color.

3.4. Production of 80% Oleic Acid Concentration with a FAD2-1A W293STOP Allele in PE529

The F_2 progenies were obtained from population three, where *FAD2-1A* W293STOP and *FAD2-1B* P137R alleles were segregated (Figure 3). Results showed that 250 individuals belonging to nine different genotypic groups were found, as demonstrated by the combination of the *FAD2-1A* and *FAD2-1B* alleles. Progeny lines with functional alleles of *FAD2-1A* and *FAD2-1B* genes produced the lowest oleic acid concentration (17.4%), resulting in transgressive segregation. In contrast, individuals with *fad2-1a* W293STOP and *fad2-1b* P137R alleles had an average of 80.5% oleic acid concentration, which was significantly higher than those of other genotypic groups (Figure 3). The oleic acid concen-

tration ranged from 72.0–84.1% in individuals with a combination of *fad2-1a* W293STOP and *fad2-1b* P137R alleles.

The F_2 progenies, where *FAD2-1A* W293STOP, *FAD2-1A* S117N, and *FAD2-1B* P137R alleles were segregated, were obtained from population four (Figure 4). In population four, the soybean cultivar Hosim produced approximately 80% of oleic acid concentration, which also contained both mutant alleles of *FAD2-1A* S117N from 17D and *FAD2-1B* P137R from PI 283327. Based on the 288 F_2 seeds determined for their fatty acid profiles, 78 produced more than 70.0% oleic acid concentration and were genotyped with molecular markers for *FAD2-1A* S117N, *FAD2-1A* W293STOP, and *FAD2-1B* P137R mutant alleles (Figure 4). A total of 78 F_2 progeny lines also had a homozygous mutant allele of the *FAD2-1B* gene (P137R), which were segregated with *FAD2-1A* S117N and *FAD2-1A* W293STOP alleles. The oleic acid concentration of the F_2 progeny ranged from 71.8–79.9% for homozygous *fad2-1a* S117N/*fad2-1a* S117N, 77.1–83.7% for heterozygous *fad2-1a* S117N/*fad2-1a* W293STOP, and 78.6–83.2% for homozygous *fad2-1a* W293STOP/*fad2-1a* W293STOP. Progeny lines with *fad2-1a* W293STOP and *fad2-1b* P137R alleles produced significantly higher oleic acid concentrations (81.1%) than other genotypes (Figure 4). Hence, these results indicate that the *fad2-1a* W293STOP allele from PE529 can produce oleic acid concentrations of greater than 70% when combined with the mutant allele of the *FAD2-1B* gene.

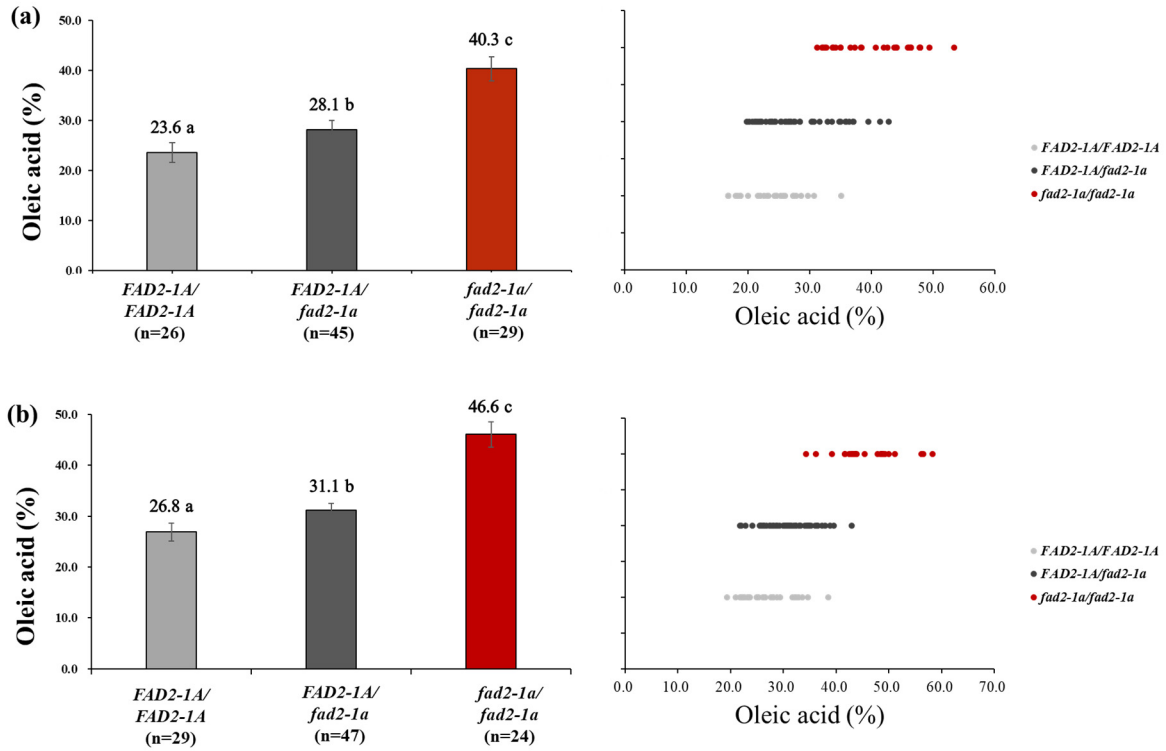

Figure 2. Distribution of oleic acid content from two segregating populations. (**a**) 100 F_2 seeds from a cross of PE529 and Pungsannamul showed altered oleic acid based on its genotype of *FAD2-1A* gene. (**b**) 100 F_2 seeds from a cross of PE529 and Sunpung showed altered oleic acid based on its genotype of *FAD2-1A* gene. The letters above the bars are statistically different based on least square difference. The *FAD2-1A* indicates functional microsomal delta-12 fatty acid desaturase 2 gene which is similar to the Williams 82 reference sequence for *Glyma.10G278000*. The *fad2-1a* indicates mutant allele of *FAD2-1A* W293STOP in PE529.

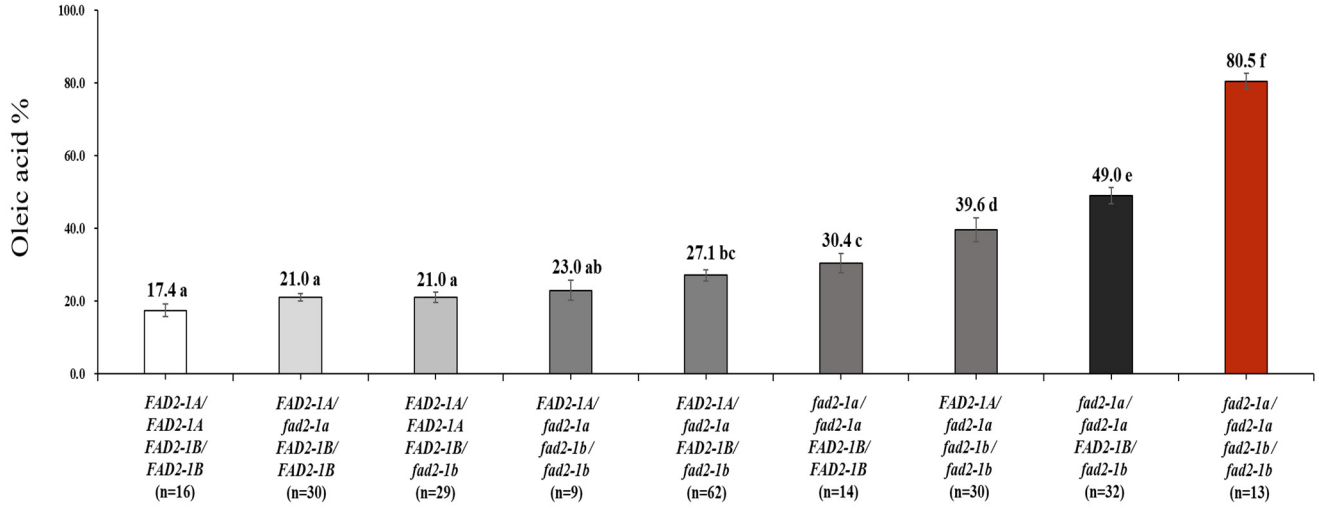

Figure 3. Distribution of oleic acid concentrations from a cross of PE529 and PI 283327 and fatty acid profiles by gas chromatography. A total of 235 F_2 seeds of the population showed altered oleic acid portion based on its genotype of *FAD2-1A* and *FAD2-1B* gene. The *FAD2-1A* and *FAD2-1B* indicates functional microsomal delta-12 fatty acid desaturase 2 gene which is similar to the Williams 82 reference sequence for *Glyma.10G278000* and *Glyma.20G111000*, respectively. The *fad2-1a* indicates mutant allele of *FAD2-1A* W293STOP in PE529. The *fad2-1b* represents variant in FAD2-1B gene in PI 283327 (P137R). The PE529 has a mutant allele of *FAD2-1A* gene (*fad2-1a* W293STOP) and a functional allele of *FAD2-1B* gene. PI 283327 contains a homozygous functional *FAD2-1A* gene and a *fad2-1b* P137R allele. The letters above the bars are statistically different based on least square difference.

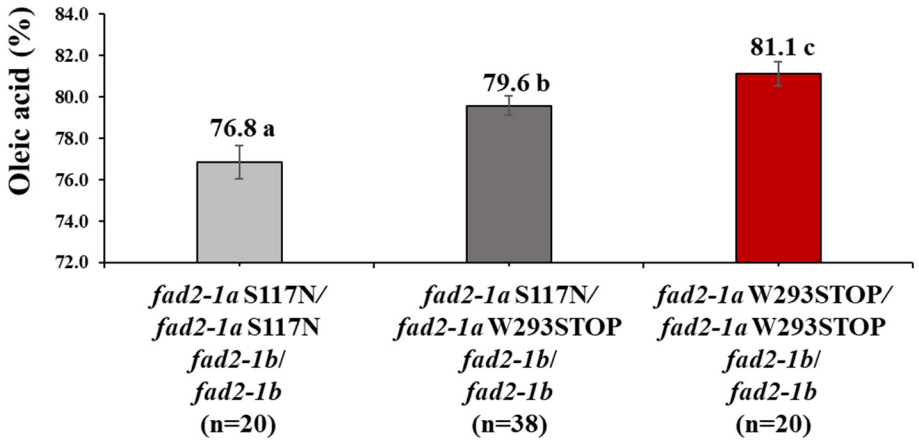

Figure 4. Oleic acid content based on the genotype of *FAD2-1* genes from a cross of Hosim and PE529. A total of 288 F_2 seeds were investigated for fatty acid profiles. Individuals with approximately 80.0% oleic acid based on gas chromatography analysis were selected. A total of 78 individuals were genotype with a *FAD2-1A* S117N allele from 17D, a *FAD2-1A* W293STOP allele from PE529, and a *FAD2-1B* P137R allele from PI 283327. The *FAD2-1A* and *FAD2-1B* indicates functional microsomal delta-12 fatty acid desaturase 2 gene which is similar to the Williams 82 reference sequence for *Glyma.10G278000* and *Glyma.20G111000*, respectively. The *fad2-1a* S117N indicates mutant allele of *FAD2-1A* in 17D. The *fad2-1a* W293STOP indicates mutant allele of *FAD2-1A* in PE529. The *fad2-1b* represents variant in *FAD2-1B* gene in PI 283327 (P137R). The PE529 has a mutant allele of *FAD2-1A* gene (*fad2-1a* W293STOP) and a functional allele of *FAD2-1B* gene. Hosim contains *fad2-1a* S117 allele and a *fad2-1b* P137R allele. The letters above the bars are statistically different based on least square difference.

4. Discussion

EMS is one of the chemicals that induce mutations in plant genomes to generate mutagenic plants. In this study, which mainly focused on mutant lines with elevated oleic acid concentrations, seed fatty acid concentrations in a mutation population consisting of 2281 lines (Figure S1) were evaluated. Considering that the role of FAD2 in soybean was to elevate oleic acid concentrations, the *FAD2-1A* and *FAD2-1B* genes with 48 elevated oleic acid mutant lines were sequenced from a mutation population using a reverse genetic approach [12,26,32]. Here, a new allele of *FAD2-1A* in the EMS-induced line PE529 was identified (Figure 1). However, no new mutation in the *FAD2-1B* gene from the 48 mutant lines with elevated oleic acid concentrations was identified. This finding was found to be in contrast to that of Comb and Bilyeu [10], which reported two novel alleles of the *FAD2-1A* gene from 2,431 EMS mutant lines and no mutations in the *FAD2-1B* gene. In our study, we identified 163 EMS mutant lines from 2281 lines with altered fatty acid profiles, including 48 elevated oleic acid mutant lines. Of 163 mutant lines with altered fatty acid profiles, PE1544 and PE1604 from 2281 mutant lines had a novel allele of the *KASII-A* gene, resulting in elevated 16:0 fatty acid (data not shown). Thus, 163 mutant lines may potentially contain a new allele of genes that are responsible for fatty acid biosynthetic pathways. Hence, further research on the sequencing of these candidate genes is necessary. The 163 mutant lines identified in this study may be used as valuable genetic resources for fatty acid studies in soybean breeding programs in the future.

The sequencing of pooled PCR amplicons from elevated oleic acid EMS-induced lines also revealed a new allele of *FAD2-1A* in PE529. PE529 contained a nonsense mutation that carried an SNP (G50014969A) in a coding region, resulting in the substitution of the amino acid tryptophan (TGG) with a stop codon (TGA) at 293 amino acids (W293STOP). As the most induced mutations for EMS treatment were C/G to A/T SNPs in plants, the *fad2-1a* W293STOP allele is a typical transition mutation (G50014969A). For the inheritance of the *FAD2-1A* W293STOP allele, two segregating populations with PE529 were developed in this study. Progeny individuals containing homozygous *fad2-1a* W293STOP alleles from the two segregating populations produced significantly higher levels of oleic acid than other genotypes such as the homozygous and heterozygous *FAD2-1A* alleles (Figure 2). Hence, the *fad2-1a* W293STOP allele is inferred to be primarily responsible for elevating the oleic acid concentrations in PE529. In addition, a single recessive allele was also found to have primary control.

To date, only 11 different mutant alleles of *FAD2-1A* containing oleic acid within the range of 27–37% were discovered in natural germplasms and mutagenized populations [9,10,37]. In several previous studies, combinations of different mutant alleles of *FAD2-1A* and *FAD2-1B* were reported to show different effects on the accumulation of oleic acid [9,23,26,32], and in a recent study by Nan et al. [38], a naturally mutated allele of *FAD2-1A* gene (W254STOP) from landrace 'Jiangsu110' with a 36.3% oleic acid concentration was identified. Authors have reported one progeny line ('435'), which can produce 91.0% oleic acid concentration in soybean seeds, in which it contained both *fad2-1a* W293STOP and *fad2-1b* P137R alleles. With this, a high-yielding soybean cultivar called Fuhang3, with 75% oleic acid concentration, was developed [38]. Bilyeu et al. [9] used three different mutant alleles of *FAD2-1A* combined with a *fad2-1b* P137R allele and observed a phenotypic variation in the oleic acid concentration. The *fad2-1a* C366R and *fad2-1a* T316I alleles also produced 81.2% and 60.3% oleic acid when combined with a *fad2-1b* P137R allele, respectively [10]. In this study, the combination of a *fad2-1a* W293STOP and a *fad2-1b* P137R allele was found to produce 80.5% oleic acid, which was more significant than progeny individuals consisting of S117N mutation in *FAD2-1A* and a *fad2-1b* P137R allele (76.8%) (Figures 3 and 4), and hence can be used in the development of soybean cultivars with high oleic acid content and high soybean yield.

The temperature during the seed-filling stage is an important environmental factor in the accumulation of fatty acids in soybean oil, except stearic acid [39]. Lee et al. [40] reported that an elevated oleic acid soybean genotype, N98-4445A, produced 46.8% oleic

acid at 26.6 °C, which was the average maximum temperature during 30 days of seed-filling stages (Columbia, MO, USA). Further, in two different locations in the USA, 55.8% of oleic acid was produced at a mean temperature of 28.1 °C (Portageville, MO USA), and 69.9% at a mean temperature of 31.8 °C (Stoneville, MS USA). In addition, it was also demonstrated that delayed planting and increasing latitudes were associated with a reduced oleic acid concentration of soybean genotypes across six environments [40]. However, several studies demonstrated otherwise, as it was found that soybean genotypes with more than 70% oleic acid concentrations displayed relatively stable development even in different environments [9,40,41]. In this study, PE529 produced 49.1% and 43.4% oleic acid concentrations in two different growing years (data not shown). However, as the progenies were not grown in different environments, further research is required to fully determine the stability of oleic acid for the *fad2-1a* W293STOP allele when combined with a *fad2-1b* allele.

In conclusion, this study identified a novel allele of *FAD2-1A* from the EMS-induced line PE529 and an SNP-induced conversion of amino acids from tryptophan to a premature stop codon at the 293 amino acid sequence (W293STOP). The novel allele can also produce soybean oil with more than 80% oleic acid concentration when combined with the *FAD2-1B* P137R mutant allele, producing a significantly higher oleic acid content than other existing *FAD2-1A* S117N mutant alleles from 17D. This study also provided evidence that molecular markers can be easily used for marker-assisted selection to detect the *FAD2-1A* W293STOP allele. Therefore, soybeans with this allele can potentially be a useful genetic resource for the improvement of soybean oil quality in breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092115/s1, Figure S1. Distribution of fatty acid concentration in the 2281 EMS mutant lines; Figure S2. Genotyping of *FAD2-1A* W293STOP allele, sequencing information, primers and simpleprobe; Table S1. Altered fatty acid profile of 48 mutant lines and wild type Pungsannamul; Table S2. Primers and the size of amplicons for the *FAD2-1A* and *FAD2-1B* genes.

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